

Circulating tumor cells: the 'leukemic phase' of solid cancers

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It is well known that malignant cells circulate in the bloodstream of patients with solid tumors. However, the biological significance of circulating tumor cells (CTCs) and the clinical relevance of their detection are still debated. Besides technical issues regarding CTCdetection methods, discontinuous shedding of CTCs from established cancer deposits, genomic instability and metastatic inefficiency might underlie the conflicting results currently available. Nevertheless, technological advances and recent clinical findings are prompting researchers to dissect CTC biology further. Here, we review these recent findings, and discuss the prospects for the identification and molecular characterization of the CTC subset that is responsible for metastasis development. This would provide a formidable tool for prognosis evaluation, anticancer-drug development and, ultimately, cancer-therapy personalization.

Biology of circulating tumor cells

Although therapeutic advances were made during the past few decades, many patients still die from metastatic cancer despite having no clinically detectable disease after treatment. In these patients, cancer recurrence originates from microscopic tumor residues known as minimal residual disease (MRD) (see Glossary). MRD can affect different body compartments, including the bone marrow, lymph nodes and peripheral blood [1–3]. The search for MRD in the peripheral blood is performed routinely for the therapeutic management of patients with hematological malignancies because the bloodstream is the physiological milieu for this kind of tumor. By contrast, the biological significance of circulating tumor cells (CTCs) in solid cancers is still debated.

The idea to investigate the metastatic process in peripheral blood originated in the 19th century when T.R. Ashworth first described the phenomenon of CTCs and S. Paget hypothesized a non-random pattern of cancer metastatization (the 'seed and soil' theory) [3,4]. Subsequently, the malignant nature of CTCs was confirmed by demonstrating that they possess tumor-specific chromosomal aberrations [5,6] and, *ex vivo*, that they grow as cell lines with malignant phenotype [7]. From model systems, it has been estimated that $\sim 1 \times 10^6$ tumor cells per gram of tumor tissue can be introduced daily into the bloodstream [8]. In cancer patients, CTCs can reach the peripheral blood from persisting dormant tumor deposits every few hours and can remain there for as long as 22 years [9]. However, a significant percentage of CTC is apoptotic, and thus might be unable to settle in secondary organs [10]. Moreover, as stated by the 'seed and soil' theory, tumors contain genetically heterogeneous cell subpopulations with different metastatic potential, which depends on the expression of relevant molecules (Figure 1). These requirements explain why the presence of CTCs is necessary but not sufficient for the metastatic process to occur. This phenomenon, known as metastatic inefficiency, has been demonstrated extensively in preclinical models [11] and represents a major issue when CTCs are used as markers of MRD in solid tumors.

Until recently, CTC biology has been neglected because most cancer research has focused on the microenvironmental features of primary tumors and established metastases. During the past ten years, accumulating

Glossary

 $[\]beta$ -type error: the statistical error (also known as type-II error) made in testing an hypothesis when it is concluded that an intervention (or prediction) is not effective (or true) but it really is.

Cell-enrichment methods: any biotechnology aimed at sorting target cells (e.g. CTCs) from a pool of 'unwanted' cells (e.g. peripheral mononucleated cells) to increase the number of cells of interest per unit of volume (enrichment) and thus enable collection of enough biological material for molecular analysis.

Genomic instability: the pathological tendency of the genome of some cells (typically malignant cells) to undergo molecular alterations (e.g. gene mutations or deletions and chromosomal translocation or deletions). Genomic instability has a key role in cancer development and progression.

Illegitimate transcription: the low-level presence of any transcripts in any cell. Accordingly, using PCR, illegitimate mRNA of any gene can be potentially amplified from any tissue or cell type.

Metastatic inefficiency: the phenomenon by which only a subset of malignant cells can metastasize because of its metastatic molecular profile.

Minimal residual disease: the microscopic tumor remnants that can persist after apparently radical treatments and that can cause disease recurrence. It is detectable only with cytometric or molecular methods (but not with currently available radiology imaging).

Pseudogene: a sequence of DNA that is similar to a normal gene but that has been altered slightly so it is not expressed. Such genes were probably once functional but, during evolution, have acquired one or more mutations that rendered them unable to produce a protein product.

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Figure 1. According to the 'seed and soil' theory, the metastatic process is largely inefficient and the fates of circulating tumor cells (CTCs) can vary according to their molecular profile. To generate a metastatic deposit, CTCs should: (i) express appropriate survival or anti-apoptosis factors [e.g. survivin, telomerase, epidermal growth factor receptor (EGFR) and Bcl-2], adhesion or homing factors [e.g. integrins, focal-adhesion-kinases (FAK), cadherins and laminins], invasion factors [e.g. matrix metallo-proteinases (MMP) and urokinase plasminogen activator (uPA)] and angiogenic factors [e.g. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), inducible nitric oxide synthase (iNOS) and hypoxia inducible factor (HIF)]; (ii) avoid anoikis [i.e. anchorage-dependent cell death occurring when cell adherence to the extracellular matrix (ECM) is lost]; (iii) resist shear forces; and (iv) escape immune surveillance. These considerations have important implications in the study of the prognostic value of CTC detection. Abbreviation: PBMC, peripheral-blood mononucleated cell.

evidence has shown that the presence of CTCs in the peripheral blood of patients with solid malignancies correlates with clinical outcome; however, some results are still conflicting and further research is needed before CTC detection can be implemented into the routine clinical setting. If the CTC subset responsible for metastasis development was identified and characterized at the molecular level, researchers and oncologists would have a revolutionary tool to tailor cancer treatment on a single-patient basis. Indeed, their investigations and clinical decisions would be centered on the biological entity (i.e. CTCs with metastatic potential) responsible for disease progression (Box 1). Here, we review the methods and results of CTC detection in patients with solid tumors and discuss the current limitations and potential developments in this field of cancer research.

CTC detection

Several approaches to detect CTCs have been described (Figure 2) and can be classified into PCR-based and cytometric methods. Because of the lack of comparative investigations, no ideal technique is available and many issues must be addressed when searching for CTCs.

PCR-based methods

PCR-based methods are the most-widely employed techniques for CTC detection. Specificity is achieved by designing the oligonucleotide primers, which are sequences specific for the gene(s) of interest. Using PCRbased methods, the expression of tumor- or tissue-specific genes and the presence of genetic abnormalities can be detected in a clinical specimen with higher sensitivity (one malignant cell out of 10^6-10^7 normal cells, which corresponds approximately to one malignant cell in 1–10 ml of blood) than that of other techniques such as light microscopy (one malignant cell out of $10^2 - 10^3$ normal cells) and immunocytochemistry (ICC) (one malignant cell out of 10^4 – 10^5 normal cells) [12], at least when cellenrichment methods are not used. PCR-based analysis also offers a high level of efficiency: the total genomic DNA or RNA from a clinical sample can be extracted and tested in a single reaction. Standard PCR (one pair of primers) and nested PCR (two pairs of primers: one pair amplifies a nucleotide sequence that is internal to the amplicon obtained with the other pair) are qualitative methods but semi-quantitative results can be obtained by competitive PCR and by ELISA (enzyme-linked immunosorbent assay) or Southern-blot detection of submaximal PCR products. Paradoxically, the main limitation of PCR is its sensitivity, which can reveal the expression of illegitimate transcripts in peripheral-blood leukocytes [13] or the presence of mRNA in normal cells that circulate at a low frequency (e.g. epithelial cells) or that contaminate blood samples during venipuncture (e.g. melanocytes,

Box 1. Why is it important to study the biology of circulating tumor cells?

Cancer biometrics - the identification of novel and more-reliable prognostic and predictive factors - is one of the most active fields in oncology [80]. Current prognostic systems (e.g. the TNM-staging system, primary-tumor molecular features and serum biomarkers) are inadequate for an optimal therapeutic management of cancer patients, as exemplified by the fact that many patients currently considered as not eligible for adjuvant therapy (because of lack of evidence of residual disease according to conventional staging methods) experience disease recurrence, whereas other patients currently submitted to adjuvant therapy (based on current prognostic systems) have only a low risk of disease recurrence (i.e. only some of them can benefit from the complementary treatment) [81,82]. Furthermore, no reliable method exists to predict sensitivity to most therapeutic regimens, which are currently administered without knowing their activity in an individual. With regard to adjuvant treatments, efficacy can be assessed only in large-scale clinical trials after an observation period of at least five years, which makes the progress in this field extremely slow.

The detection of circulating tumor cells (CTCs) is an attractive strategy for tailoring cancer treatment on a single-patient basis for the following main reasons:

keratinocytes, fibroblasts and endothelial cells). With the advent of quantitative real-time (qrt)PCR techniques [14], precise quantification of a target sequence is possible. Quantitative PCR provides investigators with not only (i) CTC presence is the necessary (although not sufficient) step in the metastatic process of solid malignancies. Accordingly, any advance in the knowledge of CTC biology might lead to significant progress in both anticancer drug development and better definition of patient prognosis.

(ii) Instead of adding another surrogate prognostic marker to the already existing plethora of such factors, CTC detection might represent an ideal prognostic tool given the active role of these cells in the metastatic process.

(iii) Because metastatic cells can have a very different molecular profile from the tumor of origin, sensitivity to conventional chemotherapeutic drugs and molecularly targeted anticancer agents might be better tested in CTCs than in the primary tumor.

(iv) If the prognostic value were confirmed, CTC detection might be of great importance for the conduction of adjuvant-therapy trials by identifying patients who need treatment (those with prognostically relevant CTCs in their peripheral blood) and by accelerating the evaluation of treatment efficacy (CTC clearance during treatment).

(v) CTCs are detected in peripheral-blood samples, which are easy to obtain (with minimal discomfort for patients) compared with other sites where minimal residual disease might be present (e.g. the bone marrow and lymph nodes).

technical (Figure 2) but also applicative advantages, such as the definition of cut-off values indicating mRNA expression levels of clinical relevance in healthy subjects compared with cancer patients, and the possibility of



Figure 2. Circulating tumor cells (CTCs) can be detected in peripheral-blood samples by two types of techniques: those based on the extraction of nucleic acids (left side, yellow) and those based on cytometric methods (right side, green). CTC molecular characterization can be performed using recently implemented innovative biotechnologies. After RNA and DNA extraction, nucleic acids can be used for PCR-based gene detection. Unlike conventional PCR, quantitative real-time (qrt)-PCR enables quantitation of target expression, does not require further assessment of results (e.g. Southern blot) and guarantees a control of mRNA quality for each sample. Alternatively, mRNA can be amplified linearly to be used for gene-expression profiling by high-throughput microarray, whereas DNA can be amplified exponentially (whole-genome amplification, particularly useful when dealing with hypocellular specimens or single cells) using linker-adaptor PCR for comparative genomic hybridization (CGH) studies. Cytometric methods enable morphological identification of CTCs, but require cell enrichment to be feasible. CTCs spun onto a slide for immunocytochemistry (by manual or automated microscopy) or fluorescence *in situ* hybridization (FISH) cytogenetic studies can be selected by laser-capture microdissection and used for CGH. Besides detecting CTCs, fluorescence-activated cell sorting (FACS) can be employed to sort cells when they are still viable and, thus, is suitable not only for gene or protein profiling but also for cell-culturing purposes.

correlating the target-sequence load with clinical outcome [15] or response to therapy [16].

DNA as a PCR target

For CTC-detection purposes, PCR targets must express tumor-specific DNA or mRNA sequences consistently. The most important advantages of using DNA as a PCR target are its stability and the independence of its abnormalities from the transcriptional activity of tumor cells. However, the disadvantages are the lack of sensitivity (one tumor cell usually contains only a single copy of the target gene) and the inability of PCR analysis to distinguish between DNA deriving from viable CTCs and free DNA shed from dying malignant cells. Moreover, unlike hematological malignancies, only few chromosomal translocations [e.g. t(11;22)(q24;q12) in Ewing's sarcoma] or gene mutations (e.g. RAS in gastrointestinal carcinomas and p53 in several tumor types) in solid tumors have sufficient specificity and frequency to be suitable for CTC detection. Because most of these studies are based on plasma samples (e.g. free DNA) [17], the use of DNA as a PCR target for CTC detection will not be discussed further in this review.

mRNA as a PCR target

Reverse transcription PCR (RT-PCR) is the most frequently used method for CTC detection. Following cDNA synthesis, the gene of interest is amplified using primers that are specific for the target gene. To avoid amplification of genomic DNA, which could potentially contaminate the cDNA preparation, these primers are either designed so that one is interrupted by an intron in the genomic DNA sequence (the intron is deleted during RNA processing, and thus does not interrupt the primer sequence in the cDNA version of the gene) or designed so that they flank an intron in the genomic sequence (thereby making it easy to differentiate between a PCR product derived from genomic DNA and another derived from cDNA, based on the size of the amplicon). The target mRNA usually encodes differentiation antigens that are expressed only in tumor cells and their parent tissues [e.g. prostate-specific antigen (PSA) in prostate carcinoma and tyrosinase in cutaneous melanoma]. Other genes, including those encoding the oncofetal proteins carcinoembryonic antigen (CEA) and a-fetoprotein, are highly expressed in tumor cells but are expressed at very low or even undetectable levels in normal cells from the same tissue. Finally, tumorspecific fusion genes [e.g. EWS/FLI-1 fusion oncogene, which results from chromosomal translocations t(11;22)(q24;q12)] and mutated genes [e.g. von Hippel-Lindau (VHL) tumor suppressor gene] are transcribed consistently by some solid tumors and have been used as markers for seeking CTCs [18,19].

Compared with DNA-based PCR, the RNA-based approach has the advantage of detecting primarily viable cells (RNA released by dying cells is rapidly degraded by ubiquitous RNases), although detection of cells in the early stages of apoptosis is possible [10], and free mRNA that encode tumor-related antigens have been found in the plasma of cancer patients [20]. A drawback of this method is that the number of mRNA copies of a gene in a given tumor cell might vary during the life cycle of the cell or as a result of de-differentiation, which might affect both standard PCR positivity and target levels detected by qrt-PCR. This phenomenon can make it difficult to interpret PCR results and distinguish between changes in tumor-cell numbers and mRNA expression levels. One possibility to circumvent this serious problem is the use of a multimarker PCR test with independent mRNA targets from different gene families [15].

Cytometric methods

Cytometric approaches, which isolate and enumerate individual cells, were the first methods employed for the detection of CTCs in the peripheral blood and currently represent the standard approach for the identification of MRD in the bone marrow. An advantage of cytometric methods is that they enable both morphological identification of malignant phenotype (which is impossible with PCR-based methods because cells are lysed to extract DNA or RNA) and further molecular characterization on a single-cell level. However, standard light microscopy and ICC sensitivity are intrinsically limited by the low frequency of CTCs in the peripheral blood, which makes the task of the observer cumbersome, time-consuming and error-prone. Nevertheless, the implementation of technological advances (e.g. automated cellular imaging and cell-enrichment methods) is renewing the interest in cytometric approaches for CTC detection [21]. Digital microscopy [e.g. automated ICC platforms and fluorescence-based laser scanning cytometry (LSC)] enables the automatic screening of blood samples on the basis of nuclear features (positivity for nucleic-acid staining) and cell-surface features (positivity for epithelial-cell antigens and/or negativity for leukocyte antigens): therefore, the operator has only the task of validating the identity of the sorted cells. Fluorescence-activated flow cytometry (FACS) is a well-known technology that is commonly used in the hematological field. In addition to an antigenical and morphometrical characterization of the cells present in a given sample, FACS technology enables the sorting of the cells of interest, which can maintain their viability and be expanded in vitro for functional studies (Figure 2).

Cell-enrichment methods

Although red-blood-cell lysis and density-gradient cell separation can be considered as cell-enrichment methods, these procedures leave CTCs largely outnumbered by peripheral-blood leukocytes. Filters with pores that enable smaller leukocytes but not the larger tumor cells to pass through are available, but most investigators using the cytometric approach for CTC detection currently rely on immunomagnetic cell enrichment (IMCE), which includes magnetic microbead system and a ferrofluid-based system. The magnetic microbead system employs antibodies (with affinity for tumor-specific or tissue-specific cell-surface markers) linked to small paramagnetic microbeads that enable target-cell selection using a powerful magnet. Commercially available microbeads are linked to antibodies that are specific for

Table	1.	Exampl	es of	studies	evaluating	the ir	npact	of C	TCs	on th	e clinica	l outcome	of pa	tients	with a	solio	l ma	lignanci	es ^{a,b}
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Tumor type	TNM stage	Number of	CTC-detection	Tumor	% CTC	Impact on	Refs
		patients	method	marker(s)	positivity	survival	
Melanoma	II, III	111	PCR	Tyrosinase	45	OS, PFS: yes	[32]
Melanoma	II–IV	118	PCR	Tyrosinase, gp100, MART1, MAGE3	47	PFS: yes	[65]
Melanoma	III	110	PCR	Tyrosinase	49	PFS: yes	[33]
Melanoma	II–IV	46	PCR	Tyrosinase, p97, MAGE3, MUC18,	96	PFS: yes	[66]
Melanoma	1–111	186	PCR	Tyrosinase, MART1	43	PFS: yes	[67]
Melanoma	II–IV	73	PCR	Tyrosinase	1	OS: yes	[68]
Melanoma	11, 111	40	PCR	Tyrosinase, MART1	70	PFS: yes	[29]
Melanoma	I–IV	212	PCR	Tyrosinase	22	OS, PFS: yes	[69]
Melanoma	111	30	PCR	Tyrosinase, MART1, uMAG-A	37	OS, PFS: yes	[34]
Melanoma	I–IV	164	Cytometric	*	26	OS: yes	[5]
Melanoma	IV	85	PCR	Tyrosinase, MART1	39	OS: yes	[70]
Melanoma	I–IV	200	PCR	Tyrosinase, MART1, p97	81	PFS (I–III): yes PFS (I–IV): no	[71]
Melanoma	II, III	60	PCR	Tyrosinase	70	PFS: yes OS: no	[72]
Melanoma	II–IV	120	PCR	Tyrosinase	44	OS: yes PFS: no	[36]
Breast	I–IV	198	PCR	Cytokeratin	33	OS, PFS: yes	[73]
Breast	IV	103	PCR	, Cytokeratin, EGP2, p1B, PS2 ^c	32	OS, PFS: yes	[15]
Breast	I, II	100	PCR	CEA	34	PFS: yes	[74]
Breast	I–III	100	PCR	Cytokeratin	33	PFS: yes	[75]
Breast	IV	177	Cytometric	Cytokeratin	49	OS, PFS: yes	[37]
Breast	I–IV	114	Cytometric	Cytokeratin	25	PFS: no	[76]
Prostate	III	30	PCR	PSA, PSMA	43	PFS: yes	[46]
Prostate	1–111	319	PCR	PSA	27	PFS: no	[44]
Prostate	I, II	145	PCR	PSA	27	PFS: yes	[43]
Prostate	IV	162	PCR	PSA	44	OS, PFS: yes	[77]
Prostate	IV	37	Cytometric	Cytokeratin	62	OS: yes	[38]
Prostate	I–III	141	PCR	PSA, PSMA	52	PFS: no	[78]
Colorectal	IV	37	PCR	Cytokeratin	46	PFS: yes	[49]
Colorectal	I–III	66	PCR	CEA	77	OS, PFS: no	[79]
Colorectal	I–IV	52	PCR	CEA, cytokeratin	15	OS: no	[50]

^aSelection criteria were number of patients in the study (\geq 30) and type of survival analysis (multivariate Cox proportional hazard model).

^bAbbreviations: CEA, carcinoembryonic antigen; EGP2, epithelial glycoprotein-2 (Ep-CAM); MAGE3, melanoma-associated antigen 3; MART1, melanoma antigen recognized by T cells-1; MUC18, melanoma cell adhesion molecule; OS, overall survival; PFS, progression-free survival; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen; uMAG-A, universal melanoma antigen gene-A.

^cPS2 is a human breast cancer prognostic marker.

^{*}In this study, morphology only (light microscopy) was used to identify circulating tumor cells.

positive selection (e.g. anti-cytokeratin monoclonal antibody for CTCs of epithelial origin) or to anti-CD45 antibodies for leukocyte depletion. The ferrofluid-based system uses anti-EpCAM (epithelial-cell adhesion molecule) antibodies coupled to 1-µm colloids (ferrofluids) followed by magnetic separation. Because most solid cancers are of epithelial origin, the use of antibodies that target surface antigens (e.g. cytokeratins) that are shared by epithelial cells (both normal and malignant) has rapidly become the most common way to sort CTCs from peripheral-blood samples before cytometric analysis [22]. However, the lack of widely expressed markers on the cell surface of non-epithelial solid malignancies (e.g. melanoma and sarcomas) does not enable the application of this method to all types of cancer. Although the implementation of cell-enrichment methods has renewed the interest in cytometric methods, this technology presents a limitation in the potential loss of CTCs during the enrichment steps; whether this cell loss has a negative impact in detecting prognostically informative CTC levels has to be elucidated.

Clinical results

Several studies have investigated the prognostic value of CTC detection in patients who have almost every type of solid malignancy (for examples, see Table 1). Because the false-positive rate among control subjects (i.e. healthy subjects or patients with non-malignant diseases) is extremely low, the specificity of both PCR-based and cytometric methods is ~100% [23,24]. Nevertheless, because there are some conflicting results, no definitive conclusion on CTC biological significance in solid tumors is available yet.

Interpretation of clinical findings is particularly challenging because published data have a high degree of variability in terms of:

- tumor type; stage of disease; timing (i.e. before, during or after treatment; with versus without evidence of disease) and number of blood withdrawals;
- methods of CTC detection (PCR-based versus cytometric); technical features (e.g. density-gradient cell separation, red-blood-cells lysis or whole blood; with

versus without cell enrichment; different PCR primers for the same marker; different PCR and cytometry types); type and number of tumor markers analyzed;

• clinical endpoint (correlation with disease stage or survival); statistical analysis (univariate- versus multivariate-survival analysis).

Therefore, it is evident that the standardization of protocols and the conduction of large multicentric trials are urgently needed.

Melanoma

The largest knowledge on CTC detection has been gained in patients affected by melanoma (the first solid tumor for which CTCs were detected by PCR [25]), followed by patients affected by breast, prostate and colorectal carcinoma. In melanoma patients, a correlation between CTCs and stage and clinical outcome has been reported by many, although others have observed opposite results (Table 1). The wide range of positivity (95% confidence interval: 19-94%) reported in patients with distant melanoma metastasis (stage IV) coupled with considerable differences in technical protocols and quality controls has questioned the reliability of CTC detection. Genomic instability, which is typical of most metastatic tumors [26], discontinuous shedding of CTCs into the bloodstream and low frequency of CTCs (close to the detection limit of PCR methods) have been advocated as the most likely biological variables underlying these heterogeneous results. Therefore, it has been suggested that multiple tumor markers and sequential blood samples might improve the reliability of CTC detection [15,21,27-30].

The probability of disease progression and, supposedly, of CTC detection is lowest at stage I of metastasis. In stage IV, genomic instability might undermine the expression of the tumor marker used for CTC detection. Thus, some investigators have focused on the prognostic value of CTCs in intermediate stages (stage II and III) and reported positive results [31–34]. These findings are of particular interest because novel prognostic factors for proving or confuting the efficacy of adjuvant therapies (e.g. interferon- α) for which no general consensus has been reached are urgently required.

Some reports note that CTC detection compares unfavorably with more traditional plasma markers (e.g. S-100) [35]; however, others observe that PCR positivity for tyrosinase can be of prognostic value in patients who are negative for plasma markers [36].

Breast cancer

In contrast to melanoma, many studies of breast cancer have been performed using cytometric methods, which is mainly owing to the availability of anti-epithelial-cell antibodies that are suitable for IMCE. These studies have demonstrated that CTCs can be thousands per ml of peripheral blood and, more importantly, that the criterion of presence or absence of CTCs in the peripheral blood is probably inadequate to be of prognostic value. In fact, among patients with metastatic disease, only a cut-off number of CTCs (a minimum number of CTCs above which the result of the test is considered positive or clinically valuable) per ml of blood enables identification of those with a worse clinical outcome [37]. These findings have been supported by studies using both cytometric [5,38] and PCR-based (e.g. qrt-PCR) methods [15,39,40] in patients with solid tumors other than breast cancer, such as melanoma, prostate and renal cell carcinoma.

Encouragingly, the results from preliminary studies of breast-cancer patients are mostly positive in terms of correlation with clinical outcome [22] (Table 1). Moreover, investigators have observed that, in patients with metastatic disease, CTC clearance correlates with response to both standard chemotherapy [16] and molecular-targeted therapy (e.g. trastuzumab antibody in HER-2/neu-overexpressing tumors [41]). This could lead to the early recognition of tumor sensitivity to a given therapeutic regimen not only for metastatic treatments (where tumor response can take some months before it is detectable by imaging techniques) but also for adjuvant treatments (where demonstration of efficacy usually requires years of follow-up) (Box 1).

Prostate cancer

Most scientists have adopted PCR-based methods for the study of prostate cancer, although the cytometric approach has also been successfully implemented [38]. The prognostic value of CTCs has been demonstrated by some studies but questioned by others [42] (Table 1). This might be in part because many investigators analyze blood samples from patients who show evidence of disease (e.g. pre-operatively), in which case, detection of uninformative CTCs shed by a tumor without metastatic potential is more likely. Accordingly, when pre- and post-operative CTC detection is correlated with stage and survival, only post-operative detection shows a prognostic value at multivariate analysis [43]. Because PSA serum levels are the most reliable prostate-tumor marker currently available, some investigators have compared its prognostic value with that of CTCs but, unfortunately, results are not in agreement [44–46].

Colorectal cancer

The results obtained from patients with colorectal cancer are the most controversial. The findings of univariate analyses are conflicting [47,48], and only one study, using multivariate analysis, has shown that CTC detection independently predicts patient survival [49]. A biological reason for these discrepancies might be that the liver acts as a filter for malignant cells shed by primary colorectal carcinomas and released into the portal system; one study reporting on the prognostic value of CTCs present in the portal (but not peripheral) blood supports this hypothesis [50]. Moreover, the same study describes positive results from multivariate analysis of patients with rectal-carcinoma liver metastases, from which CTCs can be delivered into the systemic circulation avoiding the hepatic filter [50].

A technical hurdle on the way to unraveling the prognostic value of CTCs in patients with colorectal cancer might be the difficulty in determining valuable cut-offs for defining CTC positivity. This issue has been recently pinpointed by a study in which researchers, using qrt-PCR to compare colorectal-cancer patients with

Table 2. Suggestions	for assessing the	biologica	l significance and	l clinical	usefulness	of CTCs
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False-positive rate	False-negative rate	Clinical study design
Use quantitative detection methods to calculate cut-off values to distinguish healthy from diseased subjects	Use RNA preservants while obtaining blood samples to reduce RNA degradation	Study larger and prospective groups of patients with longer follow-ups to perform clinical trials with low β-type error
PCR: reduce contamination and carry-over in the laboratory	Use multiple rather than single markers to address the issue of antigen loss by malignant cells	Standardize methods so that results from different studies can be compared and multicentric trials can be carried out
Discard first ml of blood samples to reduce cell contamination during venipuncture	Use cancer survival-related markers to target antigens that cannot be lost by CTCs	Use multivariate instead of univariate anal- ysis to demonstrate the independent prognostic value of CTCs
Design PCR primers so that amplification of pseudogenes (i.e. sequences of DNA similar to target gene but non-functional) is avoided	Intermittent CTC shedding: use multiple rather than single blood samplings	Prognostic power: consider direct (e.g. disease-specific survival) instead of indirect (e.g. disease stage) end-points
Use cytometric methods to morphologically verify the identity of CTCs detected by PCR methods	Define the most appropriate timing for blood- sample withdrawal (e.g. pre-, during or post- chemotherapy)	Analyze positive and negative predictive value of CTCs in single patients and not just the survival differences between CTC- positive and CTC-negative groups
Use metastasis-related markers to detect only CTCs with metastatic potential and thus with the highest prognostic value	CTC low frequency: use cell-enrichment procedures and larger blood-sample volumes	Design trials considering clearance of CTCs as a marker of response to therapy to compare survival of patients with and with- out CTC clearance
Define the most appropriate timing for blood-sample withdrawal (e.g. pre- versus post-surgical resection)	PCR-primer design: consider gene poly- morphism and gene mutation or deletion	Compare CTCs with traditional markers (e.g. CEA, CA19.9 and S-100) and novel methods for tumor-marker (e.g. serum proteomics) and detection of minimal residual disease (e.g. molecular imaging)

healthy subjects, could not find a difference in expression of mRNA encoding the two most-common colorectalcancer markers, CEA and cytokeratin [51].

Future perspectives

Technical and biological hurdles might prevent oncologists from proving the prognostic value of CTC detection. Several steps can be taken to address these issues and improve the quality of future studies that are designed to investigate the prognostic value of CTCs in patients with solid tumors (Table 2).

Technical issues

An intrinsic limitation of CTC studies to date is the low number of patients enrolled, which raises the question of whether the studies have a type- β statistical error low enough to enable reliable assessment of CTC prognostic impact. In addition, clinical studies should be designed to yield the most-informative data (e.g. multivariate analysis to demonstrate independence of prognostic values) and to be comparable with other series (e.g. by standardization of CTC-detection techniques).

In PCR-based methods, contamination events and amplification of pseudogenes by poorly designed primers [52] can lead to high rates of false positives, whereas lowquality samples can result in false low rates of CTC detection. Quality controls have not been described clearly in many publications; however, only rigorous ones (e.g. by quantitation of housekeeping-gene expression) can lead to reliable and comparable results.

Cytometric methods are rapidly gaining momentum (particularly in the case of epithelial cancers), and have often been linked to favorable results in terms of CTC correlation with traditional prognostic factors and clinical outcome. However, the experience gained using this approach is limited and its reproducibility has not been investigated as much as that of PCR methods.

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Furthermore, when compared with PCR-based methods, the cytometric approach shows less sensitivity [53], although conflicting findings have been reported in the case of MRD in bone-marrow samples [54].

A crucial, and still unresolved, issue regards the optimal number of assays required to demonstrate whether a blood sample is positive (e.g. single, duplicate or triplicate PCR) and the interpretation of divergent results. Unfortunately, in the absence of large-scale comparative studies, no validated rational guidelines can be proposed as yet, which leaves the design of CTC studies to the personal experience and opinion of researchers making it even more difficult to compare different results. Overall, larger and more homogeneous groups of patients who are enrolled within the framework of multicentric international collaborations must be tested to resolve the outstanding questions surrounding these and other technical issues (Table 2).

Biological factors

Dealing with biological issues seems even more challenging than dealing with technical issues. Illegitimate transcription can increase the false-positive rate, although quantitative methods (both PCR-based and cytometric) can be used to set cut-off values (e.g. upper limit of the 95% confidence interval observed in control subjects).

Conversely, intermittent shedding of CTCs into the bloodstream and genomic instability of malignant cells (which underlies the loss of expression of the molecules targeted by CTC-detection methods) can lead to falsenegative results. A logical way to deal with these two issues is to obtain multiple samples from each patient and to analyze the expression of multiple markers, thereby increasing the probability of detecting CTC [15,21,27–30].

However, the phenomenon of metastatic inefficiency (i.e. not all CTCs that are detected with current methods are viable and able to settle in secondary organs) can obscure the prognostic value of a strategy that is based simply on the presence or absence of CTCs. Therefore, an ideal target gene (or protein) would be one that is stably expressed because of its essential role in cancer-cell survival or biological aggressiveness, which is not the case for almost all tumor markers employed so far. Recently, molecular targets that are related to cancercell survival or apoptosis {e.g. telomerase [55] and epidermal growth factor receptor (EGFR) [56]} and metastatic potential (e.g. prostate stem-cell antigen [57], c-Met [58] and cadherin-6 [39]) have been implemented for CTC detection and, hopefully, will provide prognostically more-informative data. In this regard, molecular characterization of CTCs might enable investigators to define the specific molecular profile of CTCs that can metastasize, which is likely associated with patient clinical outcome. Recent biotechnological developments {e.g. RNA amplification, gene microarray [59], comparative genomic hybridization (CGH) [60] and proteomics [61]} have enabled the examination of the whole-gene or protein profile not only from hypocellular samples (e.g. peripheral-blood samples after cell enrichment) but also from single cells. This might be of particular relevance for dissecting the different metastatic potential of CTCs. For example, investigators have exploited high-throughput gene microarray [62] to demonstrate that the molecular signature that differentiates CTCs from peripheral-blood leukocytes is composed of genes of unknown function, underscoring the fact that the markers currently used for CTC detection might not be the most appropriate to identify such cells efficiently. Other investigators, using single-cell CGH, have shown that CTCs (as well as other types of early-disseminated tumor cells such as those found in the bone marrow or in lymph nodes) are genomically instable. These findings, which support the 'seed and soil' theory, highlight the need for the identification of the CTC subset that is able to generate a metastatic deposit and thus provides the greatest prognostic value. Accordingly, the characterization of a single CTC on a genomic scale is being advocated as a promising approach for the rational design of targeted anticancer therapies [63].

Concluding remarks

The current evidence is that malignant cells circulate in the peripheral blood of patients with solid tumors [5-7,23]. Although the results of several studies support a correlation between CTCs and patient clinical outcome, the findings of other studies question the biological significance, and thus the clinical usefulness, of CTC detection. Because the presence of CTCs is necessary (although not sufficient) for the development of metastatic-tumor spread, researchers are prompted to investigate the potential of CTCs as non-surrogate prognostic markers further. In addition, because it is the 'avantgarde' of solid tumors, CTCs might be considered a novel target for the development of effective anticancer drugs. However, some outstanding questions remain to be answered before CTC detection becomes a tool for the routine clinical management of cancer patients (Box 2). Recently

Box 2. Outstanding questions

- Will the broader implementation of rational protocols (Table 2) and recent technologies (e.g. quantitative real-time PCR, cellenrichment methods and automated digital microscopy) improve the ability of detecting CTCs in the peripheral blood of patients with solid malignancies?
- Many investigators have reported a positive correlation between detection of CTCs and patient prognosis, although the number of patients enrolled in each single series is low (rarely > 200) or very low (<50); will the implementation of CTC detection in the biological studies of large-scale clinical trials prove the prognostic power of CTCs detected with currently available methods?
- CTCs are low-frequency cells scattered among millions of peripheral-blood mononucleated cells. Will the implementation of novel biotechnologies (e.g. high-throughput technologies, single-cell genomics and proteomics) fulfill the promise of comprehensively describing the molecular signature of CTCs, the 'avant-garde' of solid malignancies?
- Will dissection of CTC biology define the molecular profile of those CTCs that can metastasize? Will these insights enable investigators to identify an ideal marker (or set of markers) to detect only prognostically informative CTCs and/or to design more effective anticancer drugs?

implemented biotechnologies can help investigators improve both the accuracy of CTC detection and the understanding of CTC biology, which might lead to the identification of CTC subsets with metastatic potential and thus with the greatest prognostic significance. In the meantime, the biological significance and prognostic value of CTCs can only be validated in large, prospective and homogeneous groups of patients. For example, three years ago the detection of MRD was included in the TNMclassification system of breast cancer [64], although no therapeutic changes have been suggested on the basis of MRD identification by molecular methods. In light of the latest results in this field, further progress will require carefully controlled clinical trials to test the impact of CTC-based therapeutic decisions on patients' survival.

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